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Detection of *Toxoplasma gondii* in Venous Blood from AIDS Patients by Polymerase Chain Reaction

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Detection of *Toxoplasma gondii* in blood by means of the polymerase chain reaction (PCR) may facilitate the diagnosis and follow-up of cerebral toxoplasmosis in patients with AIDS. We evaluated this approach with seven patients with tissue culture-proven parasitemia, 14 patients with presumptive cerebral toxoplasmosis, and 17 healthy human immunodeficiency virus-positive controls. Each sample of blood was assayed on three different occasions by a PCR assay based on detection of the gene encoding the P30 surface protein. A positive PCR diagnosis required positivity in at least two of the three PCR tests. None of the controls had a positive PCR diagnosis, but six of the seven patients with parasitemia did. Cerebral toxoplasmosis was confirmed in 13 of the 14 patients with a presumptive diagnosis; diagnosis by PCR was positive before treatment for 9 of these 13 patients, whereas tissue culture was positive for only 1 patient. During treatment, blood samples were taken from 14 patients at regular intervals until day 12. PCR diagnosis became negative on ethidium-stained gels, but persistent signals were observed after hybridization, in some cases, for up to 12 days after initiation of therapy. PCR on venous blood could thus be a sensitive and noninvasive method for the diagnosis of cerebral and disseminated toxoplasmosis in AIDS patients and could be a potential tool for monitoring the effects of treatment.

Cerebral toxoplasmosis is one of the most common opportunistic neurological infections in patients with AIDS (22), particularly in France (1). At present, the presumptive diagnosis is based on positive tests for anti-*Toxoplasma* antibodies, suggestive clinical signs and symptoms of central nervous system dysfunction, and typical lesions on computed tomography or magnetic resonance imaging scans of the brain (2, 7, 11). The definitive diagnosis requires the detection of tachyzoites of *Toxoplasma gondii* in histologic sections of the brain, but brain biopsy is too aggressive a measure for routine use (22). In some cases, parasitemia has been evidenced at the time of or before cerebral toxoplasmosis, suggesting that brain involvement, which likely results from a reactivation of latent cysts, may also be consecutive to or concomitant with hematological spread of the parasite (8, 22). Isolation, from blood, of the parasite by tissue culture or mouse inoculation is of questionable sensitivity (7, 28), and there is a need for specific and sensitive alternatives.

Recently, polymerase chain reaction (PCR) has been used to detect *T. gondii* in various biological specimens. Several DNA targets were used for this purpose, and sensitivity was high in the diagnosis of congenital and ocular toxoplasmosis (3, 5, 12, 15). PCR has also been used to detect *Toxoplasma* infection in brain tissue (18), cerebrospinal fluid (14, 21, 25), bronchoalveolar lavage fluid (6, 26), and blood from a limited series of patients with AIDS (19). The aim of this study was to assess the possible contribution of PCR on blood samples to the diagnosis and follow-up of cerebral toxoplasmosis in patients with AIDS.

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MATERIALS AND METHODS

Patients. Thirty-eight human immunodeficiency virus-infected patients not receiving primary prophylaxis for toxoplasmosis were investigated.

We first examined retrospectively seven patients in whom *T. gondii* parasitemia had been proven by tissue culture; toxoplasmosis had been suspected on the basis of persistent, unexplained fever. Only one of these seven patients had cerebral symptoms; the other six had extracerebral toxoplasmosis, evidenced by positive cultures of bronchoalveolar lavage fluid (four cases), muscle (two cases), bone marrow (one case), and pericardial fluid (one case).

We then examined prospectively 14 hospital patients with clinical and radiological signs suggestive of cerebral toxoplasmosis. In 12 patients who were seropositive for *Toxoplasma* infection, i.e., specific immunoglobulin G antibody level >10 IU/ml, the diagnosis was confirmed by the regression of clinical and radiological signs under specific treatment with sulfadiazine and pyrimethamine. One patient with a positive *Toxoplasma* serology died of septic shock within 48 h. The remaining patient died 4 weeks later, with no improvement despite specific anti-*Toxoplasma* therapy; cerebral toxoplasmosis was unlikely because of a negative *Toxoplasma* serology and a histologic diagnosis of retro-orbital lymphoma.

The control group consisted of 12 outpatients with a positive *Toxoplasma* serology and 5 outpatients with a negative *Toxoplasma* serology. None had clinical evidence of progressive toxoplasmosis.

Methods. One blood sample was taken before starting specific therapy and, when possible, on days 2, 4, 8, and 12 of treatment; one blood sample was obtained from each of

the control patients. Ten milliliters of heparinized venous blood was mixed with an equal volume of sterile 2% dextran T 500 (Pharmacia) in physiological saline. After 15 min of bench-top sedimentation at 1 g, the erythrocyte-free supernatant containing leukocytes and platelets was collected in two separate tubes and was centrifuged at 2,000 × *g* for 10 min. The pellets were washed twice in minimal essential medium containing 50 IU of penicillin per ml and 50 μg of streptomycin per ml. One pellet was inoculated into MRC5 fibroblast cultures as described elsewhere (9, 10), and the other was kept at −20°C for PCR.

A total of 71 blood samples from 21 patients and 17 controls were studied. Twenty-one samples were obtained before treatment, and 33 sequential samples were obtained during treatment of 3 patients with proven parasitemia and 11 patients with presumptive cerebral toxoplasmosis. In addition, four bronchoalveolar lavage fluid samples and one bone marrow sample were available from patients with parasitemia. The latter samples were washed twice in minimal essential medium, and the final pellet was processed in the same way as the blood samples.

PCR was based on the detection of the gene coding for the P30 surface protein of *T. gondii*, the sequence of which has been described by Burg et al. (4). Briefly, DNA samples were extracted from the frozen cell pellets with phenol by using a conventional method (16).

A 282-bp region (positions 418 to 700) was amplified with the synthetic oligonucleotide primers 5'ACTGATGTCGT TCTTGCGATGTGGC and 5'CGTCCACCAGCTATCTTC TGCTTCA. Amplification was carried out with *Taq* DNA polymerase (Cetus) in a Perkin-Elmer Cetus thermal cycler. *Taq* polymerase (2.5 U) was used in a 100-μl reaction volume with reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.01% gelatin), 500 μM each deoxynucleoside triphosphate, 0.5 μM oligonucleotides 1 and 2, and 1 μg of purified DNA. After an initial cycle consisting of 5 min of denaturation at 94°C, 2 min of annealing at 65°C, and 40 s of extension at 72°C, the following cycles were repeated 30 times: 30 s of denaturation at 94°C, 40 s of annealing at 65°C, and 40 s of extension at 72°C. The final step consisted of 30 s of denaturation at 94°C, 40 s of annealing at 65°C, and 5 min of extension at 72°C. The amplification products (1/10 of the volume) were stained with ethidium bromide and were subjected to electrophoretic migration in 4% agarose gels. After UV examination, the gels were transferred to Hybond-N nylon membranes (Amersham) according to the manufacturer's protocol. Southern blots were analyzed with a 207-bp insert (positions 454 to 661) as a probe (5'-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase; Boehringer). The membranes were incubated in prehybridization buffer containing 5× Denhardt's solution, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) and 40 μg of salmon sperm DNA per ml and were hybridized overnight at 65°C with the labeled probe. Blots were washed at 65°C for 15 min in 2× SSC, for 30 min in 2× SSC–0.1% SDS, and for 10 min in 1.0× SSC at 65°C and then were autoradiographed with X-ray film for 24 h at −80°C. A test was considered positive if the film showed a visible signal.

The four steps of the reaction (DNA extraction, preparation of reaction media, amplification, and analysis) were carried out in four different rooms. The sensitivity of the procedure had been previously evaluated by testing serial dilutions of DNA from purified *Toxoplasma* tachyzoites: after PCR and hybridization, 1/100 of the DNA of three organisms could be detected (13). In addition, no cross-reactivity was observed in a bronchoalveolar fluid containing numerous *Pneumocystis carinii* trophozoites (13). Each set of experiments included a positive control (DNA extracted from the RH strain of *T. gondii*) and a negative control (distilled water). Each extracted DNA sample was tested on three different occasions.

TABLE 1. Results of PCR and tissue culture of blood samples in diagnosis of toxoplasmosis in 38 human immunodeficiency virus-positive patients

Diagnosis (*n*)	Patient(s)	Class of PCR test[a]	No. of patients with positive:	
			PCR diagnosis[b]	Tissue culture
Retrospective group with tissue culture-proven parasitemia (7)	1–4	3	4	4
	5[c] and 6	2	2	2
	7[c]	1	0	1
Prospective group with suspected cerebral toxoplasmosis (14)[d]	8–14	3	7	1
	15 and 16	2	2	0
	17–20	1	0	0
	21[e]	0	0	0
Controls with positive *T. gondii* serology (12)	22–24	1	0	0
	25–33	0	0	0
Controls with negative *T. gondii* serology (5)	34	1	0	0
	35–38	0	0	0

[a] Each sample was assayed three times and was classified according to the number of positive PCR tests: class 3, three positive PCR tests; class 2, two positive PCR tests; class 1, 1 positive PCR test; class 0, no positive PCR test.
[b] A positive diagnosis by PCR requires at least two positive PCR tests of the three tests performed.
[c] Tissue culture showed very few parasites.
[d] Cerebral toxoplasmosis was confirmed in 13 patients.
[e] Final diagnosis was retro-orbital lymphoma.

RESULTS

PCR was repeatedly negative in the three tests on samples from 13 of the 17 controls, regardless of their serological status for *Toxoplasma* infection. In four controls (three seropositive, one seronegative), one of the three PCR tests was positive (Table 1). Of the seven patients with tissue culture-proven parasitemia, three of three PCR tests were positive in four patients, two of three were positive in two patients, and one of three was positive in one patient (Table 1). On the basis of these results, we considered that a positive diagnosis by PCR required two of three positive results.

In the seven patients with parasitemia, diagnosis by PCR was thus considered positive in six patients. In the only sample with a PCR-negative diagnosis, tissue culture showed very few parasites (Table 1). Diagnosis by PCR was also positive for four bronchoalveolar-lavage samples and one bone marrow sample in which *T. gondii* had been cultivated; with these samples, the three PCR tests were positive.

In the 14 patients with presumptive cerebral toxoplasmosis, diagnosis by PCR on blood obtained before treatment was positive in 9 patients, whereas tissue culture was positive in only 1 patient (Table 1). In this latter case, the patient died of septic shock 48 h after admission. Both diagnosis by PCR and tissue culture were negative in the patient with retro-orbital lymphoma (patient 21).

During treatment, blood samples were taken at regular intervals until day 12 (Table 2) in 14 patients. Tissue culture (patients 1, 4, and 7) and ethidium bromide staining (patients 1, 9, 11, and 12) were always negative after day 0. In the

TABLE 2. Tissue culture and diagnosis by PCR on blood samples from 14 patients with suspected cerebral toxoplasmosis or parasitemia before and during treatment

Patient[a]	Test	Diagnosis at[b]:				
		Day 0	Day 2	Day 4	Day 8	Day 12
1	Culture	+	ND[c]	ND	−	ND
	PCR	+[d]	ND	ND	−	ND
4	Culture	+	ND	ND	ND	−
	PCR	−	ND	ND	ND	−
7	Culture	+	−	−	ND	ND
	PCR	−	+	+	ND	ND
8	Culture	−	−	−	−	ND
	PCR	+	+	+	+	ND
9	Culture	−	ND	ND	−	ND
	PCR	+[d]	ND	ND	+	ND
10	Culture	−	−	−	−	−
	PCR	+	−	−	+	−
11[e]	Culture	−	ND	−	ND	−
	PCR	+[d]	ND	+	ND	+
12	Culture	−	ND	ND	ND	−
	PCR	+[d]	ND	ND	ND	+
15	Culture	−	ND	ND	−	−
	PCR	+	ND	ND	+	−
16	Culture	−	−	ND	ND	ND
	PCR	+	+	ND	ND	ND
17	Culture	−	−	−	−	−
	PCR	−	−	−	−	−
18	Culture	−	−	−	−	−
	PCR	−	−	+	+	−
19	Culture	−	ND	−	−	−
	PCR	−	ND	−	+	−
21[f]	Culture	−	−	−	−	−
	PCR	−	−	−	−	−

[a] Patients 8 through 19 were suspected to have cerebral toxoplasmosis; patients 1 through 7 had parasitemia.
[b] Day 0 was before treatment, and days 2 through 12 are during treatment.
[c] ND, not done.
[d] Positive on ethidium bromide-stained gel.
[e] In a blood sample taken 60 days previously, tissue culture was negative and PCR was positive.
[f] Patient 21 was suspected of having cerebral toxoplasmosis, but the final diagnosis was lymphoma.

patients with toxoplasmosis, hybridization was positive in six of eight cases on day 8 and two of eight cases on day 12. Three patterns were observed: (i) positive diagnosis by PCR on day 0 and positive or negative diagnosis thereafter (patients 1, 4, 8, 9, 10, 11, 12, 15, and 16), (ii) negative diagnoses by PCR on day 0 and thereafter (patients 17 and 21), and (iii) negative diagnosis by PCR on day 0 but positive diagnosis thereafter (patients 7, 18, and 19). Interestingly, patient 11 was febrile 2 months before definitive diagnosis of cerebral toxoplasmosis; at that time, a negative computed tomography scan ruled out the diagnosis and the symptoms were attributed to pyelonephritis. However, a blood sample taken at that time gave a positive PCR diagnosis.

DISCUSSION

The results presented here suggest that PCR testing of blood samples may be a useful diagnostic approach to toxoplasmosis in patients with AIDS. In the patients with cerebral toxoplasmosis, diagnosis by PCR was more sensitive (9 of 13) than tissue culture (1 of 13).

It has been proposed that samples be assayed in duplicate or triplicate and that results be accepted only if they are repeatable (15, 17, 27). In the present study, sporadic positive signals in individual PCR tests in controls could have been due to random contamination. With our procedure, in which a positive PCR diagnosis requires at least two positive PCR results in the three assays, the PCR diagnosis was always negative for the controls.

In the eight patients with proven parasitemia (seven in the retrospective group and one in the prospective group), diagnosis by PCR was positive in seven cases. The only discrepancy involved patient 7, in whom tissue cultures of blood revealed few parasites. Among the 13 patients with cerebral toxoplasmosis, diagnosis by PCR was positive before treatment in nine cases; in one case (patient 11), PCR detected *Toxoplasma* DNA in the blood 2 months before the onset of symptomatic cerebral toxoplasmosis. Although clearly limited, this observation suggests that cerebral toxoplasmosis may result from hematological spread of the parasite rather than from a local reactivation in brain tissue. This differs from the results obtained in acute, acquired infections in immunocompetent individuals in whom parasitemia was undetectable by PCR and tissue culture (19).

The PCR diagnoses were negative before treatment in four patients with cerebral toxoplasmosis. These negative results may be related to the absence or low number of circulating parasites or parasite DNA in cases of focal neurological reactivation. In addition, the procedure used to recover leukocyte pellets may interfere with PCR sensitivity; in this study, sedimentation on dextran allowed easy recovery of most leukocytes and platelets, but we cannot exclude the possibility that parasites may have sedimented with erythrocytes. Another separation procedure, with a mixture of polysucrose and sodium diatrizoate, has been proposed; intracellular parasites were recovered with leukocytes, whereas extracellular tachyzoites mostly remained in the erythrocyte layer (17). In the case of a low number of circulating or isolated parasites, the negativity of the diagnosis could also have been due to the absence or low numbers of targets, because the P30 gene is a single-copy gene (4).

Although PCR is not a quantitative method, the different strengths of the signals on the gels and films suggest that the number of circulating parasites may differ between patients. A positive signal on the ethidium bromide-stained gel corresponds to a large number of *T. gondii* organisms. Indeed, we have previously found that a signal is seen on gels when DNA from as few as 150 parasites is present in the sample (personal results). Assuming that the sample tested by PCR (1 μg of total DNA) corresponded to 10 to 25% of the amount extracted from 5 ml of blood, the number of circulating parasites could be as high as 120 to 300/ml. The reason for the lower sensitivity of tissue culture for the demonstration of *T. gondii* in blood is unclear, because this technique yields at least 75% positivity with an inoculum of 30 bradyzoites or tachyzoites (9) and because PCR and tissue culture have similar sensitivities for detecting parasites in amniotic fluid (5, 12, 15). However, in rodent models, PCR of blood samples was more sensitive than tissue culture or inoculation of mice (17, 20). With blood samples, the number of cells used to inoculate cultures, together with inhibitory factors, may interfere with the infection of the monolayer (17, 23). In addition, fractions of parasite genome processed and discharged from infected tissues via the bloodstream could be detected by PCR but would not be infectious for tissue culture or mice.

After initiation of therapy, no positive signals were seen on ethidium bromide-stained gels. However, the signals after hybridization remained positive, in some cases for up to 12

days. This persistence of positive PCR results has already been observed in patients with neurosyphilis, in whom *Treponema pallidum* DNA was detectable in cerebrospinal fluid several months after effective treatment (24). In our patients, the treatment was also effective, as shown by the resolution of clinical and radiological symptoms; the presence of parasite DNA in blood may thus reflect the lack of complete eradication of *T. gondii* from infected tissues. In addition, treatment may also induce the release of parasite DNA, as suggested by the fact that diagnosis by PCR became positive in three patients 2 to 4 days after the initiation of therapy. Moreover, these results suggest that PCR could have diagnostic value after initiation of therapy.

In conclusion, PCR on blood samples appears to be a sensitive method for the diagnosis of disseminated and cerebral toxoplasmosis, and a positive PCR diagnosis could be an early marker of reactivation. In addition, this method may prove to be a useful tool for monitoring therapy. Additional studies are in progress to more accurately evaluate the time required for PCR to become negative according to the therapeutic regimen.

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